Animal Conservation

Animal Conservation. Print ISSN 1367-9430

Reduced genetic diversity and isolation of remnant ocelot populations occupying a severely fragmented landscape in southern Texas

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Keywords
conservation; microsatellites; mtDNA; *Leopardus albescens*; population structure; Felidae.

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Editor: David Reed
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Received 4 March 2011; accepted 19 May 2011

doi:10.1111/j.1469-1795.2011.00475.x

Abstract

The ocelot *Leopardus pardalis* has become a conservation priority in the US as a result of severe population decline and loss of habitat during the 20th century. Only two small populations remain in this country. Their short-term viability is threatened by the disappearance of dense thornshrub communities, human-caused mortality and demographic stochasticity. The influence these factors have on ocelot persistence must be considered to develop effective conservation initiatives. We therefore examined neutral genetic diversity and connectivity among ocelots in the US and northeastern Mexico using 25 autosomal microsatellites and a 395-bp segment of the mitochondrial control region. Genetic variation was lowest in the population occurring on Laguna Atascosa National Wildlife Refuge, Texas (autosomal microsatellite $H_E = 0.399$ and mtDNA-haplotype diversity = 0) and highest in northeastern Mexico (0.637 and 0.73, respectively), while intermediate on private lands in Willacy County, Texas (0.553 and 0.252, respectively). Significant genetic differentiation between the two Texas populations was observed, despite their close proximity (~30 km). Both populations were also significantly divergent from northeastern Mexico. The absence of any detectable gene flow implies that the human modified landscape of the Lower Rio Grande Valley in southern Texas acts as a strong barrier to ocelot movement, disrupting metapopulation dynamics and contributing to loss of diversity. As a consequence, continued genetic erosion among the Texas populations is expected. The lack of movement through the fragmented landscape also suggests it is unlikely ocelots will recolonize unoccupied habitat patches along the Lower Rio Grande and the delta interior where agriculture and urban land uses predominate. The continued rapid development will exacerbate this problem. These factors threaten the persistence of the Texas populations and limit their recovery. Translocations are necessary to link ocelot populations in the US.

Introduction

Many species exhibit metapopulation dynamics, particularly in ecosystems with discontinuously distributed habitat. A metapopulation consists of smaller, local populations that periodically undergo extinction and recolonization events, often with an influx of individuals from a larger, more stable core population (Levins, 1969; Hanski, 1999). In this system, the persistence of local populations is dependent on the relative rates of extinction and recolonization; these in turn are largely dependent on the levels of connectivity (Levins, 1969; Hanski, 1999). In areas dominated by small, fragmented habitat patches where extinctions occur frequently, only high levels of dispersal can maintain local populations.

The connectivity of populations maintained by dispersal can be reduced in a landscape fragmented by unsuitable habitat resulting from anthropogenic perturbations. In areas where dispersal is compromised, and the remaining

habitat patches are small, extirpations of local populations occur more frequently than recolonizations. Fixation of deleterious alleles and loss of adaptive alleles as a result of genetic drift and inbreeding compromise fitness and synergistically interact with demographic stochasticity to further reduce viability of populations (Hedrick & Kalinowski, 2000; Reed, 2005; Reed, Nicholas & Stratton, 2007). Genetic diversity and population connectivity are therefore of major concern when developing management strategies for threatened species occupying a fragmented landscape (Reed & Frankham, 2003; Reed, 2004; O'Grady *et al.*, 2006). Spatial and temporal patterns in variation can provide insight on movement between populations (Avise, 2004), potential for inbreeding depression (Hedrick & Kalinowski, 2000) and the likelihood that extant populations will recolonize unoccupied habitat patches.

Most felids require large habitat tracks and occur at relatively low densities; therefore, they have been particularly impacted by anthropogenic pressures including habitat loss, predator control and the fur trade (Nowell & Jackson, 1996). In North America, species such as the mountain lion *Puma concolor* have begun to recover, and others including the bobcat *Lynx rufus* remain abundant even in areas with high human densities (Nielsen & Wolf, 2001; Riley & Malecki, 2001; Pimm, Dollar & Bass, 2006; Ruell *et al.*, 2009). In contrast to these success stories, the ocelot *Leopardus pardalis albescens* is yet to make a recovery in the US and is listed as endangered by US Fish & Wildlife Service and Texas Parks & Wildlife; although, across large parts of Central and South America it is common in many Neotropical lowland ecosystems (Caso *et al.*, 2008). Historically, its northern distribution extended into Arizona, Arkansas, Louisiana and Texas within the US (Murray & Gardner, 1997). Removal of dense cover and other anthropogenic factors caused major population reductions during the 20th century (Tewes and Everett, 1986). Currently, within the US the ocelot persists in only two, small populations inhabiting a severely fragmented landscape in southern Texas (Haines *et al.*, 2005; 2006*b*; Janečka *et al.*, 2008).

Ocelots in Texas are closely associated with remnant dense thornshrub communities (Shindle & Tewes, 1998; Harveson *et al.*, 2004; Jackson & Zimmerman, 2005; Horne *et al.*, 2009). One of the populations consists of 20–40 individuals occupying the Laguna Atascosa National Wildlife Refuge (LANWR) in Cameron Country (Haines *et al.*, 2005; Janečka *et al.*, 2008). The other one occurs on private lands in Willacy Country ~30 km northwest of LANWR (Haines *et al.*, 2006*a*,*b*). Janečka *et al.* (2008) estimated the effective size (N_E) of this population to be smaller than in LANWR ($N_E = 3$ compared with $N_E = 14$–16, respectively).

The two areas appear isolated, and previous studies have shown low diversity in both mitochondrial and microsatellite markers, particularly in LANWR (Walker, 1997; Janečka *et al.*, 2007; 2008). The nearest known ocelot populations are ~150 km to the south in the State of Tamaulipas, Mexico and are believed to be larger and less fragmented (Caso, 1994; Janečka *et al.*, 2007). Currently, they are separated by high human densities, widespread development and extensive croplands, particularly in the Rio Grande delta. Previous phylogenetic analysis has shown a very close relationship between Tamaulipas and Texas populations, both of which are classified within the subspecies *L. p. albescens* (Murray & Gardner, 1997; Janečka *et al.*, 2007).

Population viability analysis suggests high probability of extinction in LANWR ($P = 0.65$ within 100 years; Haines *et al.*, 2005). However, the risk was lower when the two US populations were considered in a metapopulation model ($P = 0.15$ within 50 years; Haines *et al.*, 2006*c*). The positive relationship between viability and dispersal is mediated through both demographic rescue and genetic exchange (Hanski, 1999). Therefore, the landscape connectivity has a large influence on the persistence of this species within the US, and must be incorporated with other ecological and demographic information during conservation planning.

We therefore used 25 autosomal microsatellites and a portion of the mtDNA control region to characterize genetic diversity and population structure in ocelots. Our goals were to (1) compare genetic diversity among the Texas and northeastern Mexico populations; (2) determine the degree to which fragmented populations in Texas are isolated from each other and from Mexico. These issues must be considered in order to develop effective recovery plans that ensure the persistence of the last wild Neotropical felid breeding within the US.

Methods

Study area and sample collection

All sampling sites (Fig. 1) were located in the Tamaulipas Biotic Province, which stretches from southern Texas to Tamaulipas, Mexico. This region is a transition zone between Nearctic and Neotropical fauna characterized by thornshrub, scrub forest and mixed grassland–forest habitats (Blair, 1950). Blood samples were collected from wild-caught ocelots during previous radio-telemetry studies conducted between 1986 and 2005 (Tewes, 1986; Laack, 1991; Caso, 1994; Beltran & Tewes, 1995; Shindle & Tewes, 2000; Haines, Tewes & Laack, 2005; Laack *et al.*, 2005; Haines *et al.*, 2006*a*,*b*,*c*; Horne *et al.*, 2009).

The three primary study localities included: (1) LANWR, Cameron County (i.e. Cameron, $n = 52$); (2) Yturria Ranch and other private ranches in northern Willacy County (i.e. Willacy, $n = 34$); (3) Los Ebanos Ranch, Tamaulipas (i.e. Mexico, $n = 17$; supporting information Table S1). A few additional individuals were captured in four other areas: (1) Santa Ana National Wildlife Refuge, Texas (SANWR, $n = 1$); (2) Port of Brownsville, ($n = 1$); (3) Los Zoyates Ranch, Tamaulipas ($n = 3$); (4) Miradores Ranch, Tamaulipas ($n = 3$). Ocelots that died as a result of vehicle collisions in Texas were also sampled near the following localities: Port Mansfield ($n = 6$), Raymondville ($n = 2$), Lyford ($n = 1$), Highway 186 in Willacy County ($n = 1$), Rio Hondo ($n = 1$) and Sarita ($n = 2$).

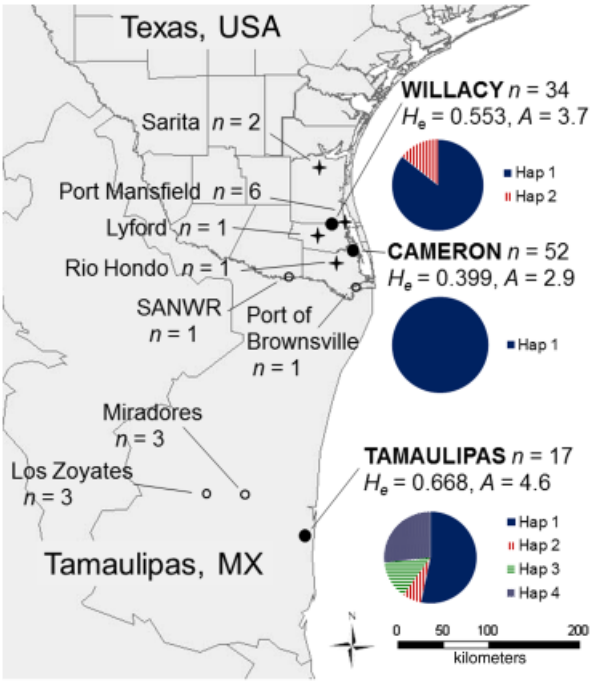

Figure 1 Map showing sampling localities in US and Mexico. Solid points represent primary populations, open circles localities outside these areas where ocelots were radio-collared, and crosses represent locations where road-killed ocelots where found. H_E = expected heterozygosity, A = mean allele number. Pie charts show mtDNA haplotype frequencies in the three populations.

Autosomal microsatellite genotyping

Blood samples were stored in lysis buffer (Longmire, Maltbie & Baker, 1997). The DNA extractions were performed with a PureGene® DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA). We initially screened 41 autosomal microsatellites isolated in the domestic cat *Felis catus* by Menotti-Raymond *et al.* (1999; 2003*a*,*b*; 2009) to select loci that would be informative in ocelots (supporting information Table S2). These were broadly distributed throughout the genome based on their position in the domestic cat radiation hybrid and linkage maps (NCBI MAP VIEWER BUILD 0.1; Menotti-Raymond *et al.*, 2003*a*; 2009; Davis *et al.*, 2009). Felids have a high level of chromosomal conservation with >95% of locus locations and order preserved across their family (Davis *et al.*, 2009). Therefore, we assumed that the genomic positions for majority of loci are shared between the ocelot and the domestic cat. Of the loci screened, the 31 that exhibited robust amplification and unambiguous alleles were used to genotype the sampled ocelots (supporting information Table S2).

We used two methods for labeling PCR amplicons. The first 15 microsatellites were genotyped using forward primers 5′-labeled with a fluorescent dye (FAM, HEX, or TET; supporting information Table S2). Reaction mixes contained 0.2 mM of each dNTP, 1 × PCR HotMaster™ *Taq* buffer (Eppendorf, Hamburg, Germany) with 2.5 mM $MgCl_2$, 0.25 units of HotMaster™ *Taq*, 0.24 mM of each primer and 20 ng of DNA template. The PCR conditions included an initial denaturing step of 94 °C for 60 s, 30 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 60 s, and a final extension step of 72 °C for 2 min. The m13 primer dye-labeling approach (Guo & Milewicz, 2003) was used for the remaining 16 loci (supporting information Table S2). Reactions contained 0.8 mM of each dNTP, 1 × PCR Platinum *Taq* buffer (Invitrogen, Carlsbad, CA, USA), 2.5 mM $MgCl_2$, 0.2 units of Platinum *Taq*, 0.027 mM forward primer with a m13 sequence tag on the 5′-end, 0.4 mM of the 5′-dye-labeled m13 primer (FAM, NED, VIC, or PET), 0.4 mM of the reverse primer, and 20 ng of DNA template. The PCR reaction conditions were 94 °C for 2 m, 40 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 60 s, and a final extension step of 72 °C for 5 min.

Direct labeled amplicons were genotyped on the ABI 3100 automated sequencer (Applied Biosystems, Forest City, CA, USA) in the Laboratory of Plant Genomics and Technology, Texas A&M University and m13-labeled amplicons on an ABI 3730 sequencer in the Equine Molecular and Cytogenetics Laboratory, Department of Veterinary Integrative Biosciences, Texas A&M University. Allele calls were made using GENOTYPER 2.0 (Applied Biosystems). Amplicons from two previously genotyped ocelot samples were included with every genotyping run to ensure alleles were consistently sized.

Y-chromosome microsatellite genotyping

There is a lack of available microsatellite loci on the Y chromosome for population genetic analysis of felids. We therefore tested 28 microsatellites discovered within introns of seven single copy genes located in the non-recombinant region of the domestic cat Y chromosome (Davis *et al.*, 2009). Primers were designed using Primer3 and PCR tested to ensure they did not amplify an X-linked paralog (Rozen & Skaletsky, 2000). The m13 primer dye-labeling PCR conditions described above were used with 1.5 mM $MgCl_2$ and 58 °C annealing temperature. Alleles were genotyped as described above. Sixteen loci were excluded because of inconsistent PCR amplification. The remaining 12 microsatellites were genotyped in 53 male ocelots (supporting information Table S1).

Mitochondrial control region sequencing

A 395-bp segment of the control region was sequenced and aligned using primers from Jae-Heup *et al.* (2001) modified to match the ocelot mitochondrial DNA sequence (PAN-OCELOT-F primer, 5′-CTCAACTATCCGAAAGAGCTT-3′, PAN-OCELOT-R primer, 5′-CCTGTGGAACATTAGGAATT-3′; Janečka *et al.*, 2007) This fragment aligns with positions 16,833 to 218 of the domestic cat mitochondrial genome, which is located in the central conserved region between repetitive sequences I and II (Lopez, Cevario & O'Brien, 1996; Jae-Heup *et al.*, 2001). The PCR

amplification was performed in 25 μL volumes containing 0.2 mM of each dNTP, 1 × JumpStart PCR buffer (Sigma Aldrich, St Louis, MO, USA), 1.25 units of JumpStart *Taq*, 0.25 mM forward primer, 0.25 mM reverse primer and 20 ng DNA template. Cycle conditions included an initial denaturing step of 94 °C for 1 min, 30 cycles of 94 °C for 15 sec, 58 °C for 30 s, 72 °C for 2 s, and a final extension step of 72 °C for 2 min. The PCR products were sequenced using an ABI BigDye v. 1.1 Terminator Kit on the ABI 3100 or ABI3730 automated sequencer following the recommendations of the manufacturer. Sequences were obtained in both directions, and contiguous sequences were constructed using SEQUENCHER 3.0.

Genetic diversity – autosomal microsatellite analysis

Measures of genetic variability, including expected heterozygosity (H_E), mean number of alleles (A) and number of private alleles (A_P), were estimated using GENALEX 6.4 (Peakall and Smouse, 2006). Allelic richness (A_R), observed heterozygosity (H_O) and gene diversity (H_S) was estimated and tested for significant differences between populations with 10 000 permutations in FSTAT 2.3.9 (Goudet, 2001). Tests for linkage disequilibrium (LD) and Hardy–Weinberg equilibrium (HWE) were performed using GENEPOP 3.1 (Guo & Thompson, 1992; Raymond & Rousset, 1995). Populations were tested for deviations from equilibrium at each locus and across all loci. The Bonferroni method was used to correct P-values for multiple comparisons in the HWE and LD tests (Rice, 1989). The three populations were tested for recent reductions in effective population sizes based on excess gene diversity across loci following the methods of Cornuet & Luikart (1996) as implemented in BOTTLENECK 1.2.02 (two-phase model and standardized differences test).

Genetic structure among ocelot populations

Samples were divided into three populations: Cameron ($n = 42$), Willacy ($n = 28$) and Mexico ($n = 12$). Pair-wise F_{st} estimates were derived and tested for significance with 10 000 permutations using the AMOVA framework in GENALEX. Effective numbers of migrants per generation (N_em) were estimated from the mean frequencies of private alleles by GENEPOP (Barton & Slatkin, 1986). One ocelot generation is *c*. 7 years based on life-history data (Janečka *et al*., 2008).

Assignment tests were conducted by estimating the probability of individuals originating from each of the populations using GeneClass 2 (Piry *et al*., 2004). The number of mis-assignments is positively related to dispersal between populations (Rannala & Mountain, 1997; Paetkau *et al*., 2004). Assignment tests were implemented using both frequency-based (Paetkau *et al*., 2004) and Bayesian methods (Rannala & Mountain, 1997). The proportion of individuals mis-assigned was compared between populations. We also tested for the presence of first generation migrants using GeneClass and STRUCTURE 2.2 (Pritchard, Stephens & Donnely, 2000). Finally, individuals sampled outside of three primary study sites were assigned to their most likely source using the three populations as reference samples in both GeneClass and STRUCTURE.

Bayesian model-based clustering in STRUCTURE was used to explore population structure without regard to geographic origin (Pritchard, Stephens & Donnely, 2000). This approach applies a Bayesian algorithm to estimate the likelihood of K clusters (synonymous with 'populations') and the portion of an individual's genetic variation (Q) attributed to each of the clusters, based on LD and HWE. We estimated the likelihood for $K = 1$–10 using the admixture model for 10 independent runs with 500 000 MCMC generations and burn-in of 100 000. The most likely number of clusters was determined using two approaches; by estimating the posterior probability for each K as recommended by Pritchard, Stephens & Donnely (2000) and by using the method of Evanno, Regnaut & Goudet (2005). The model-defined clusters were compared with the three geographically defined populations.

MtDNA data analysis

Sequence alignment was performed in SEQUENCHER and the number of variable sites, haplotype diversity (D_{hap}), nucleotide diversity (π) and departure of haplotype frequencies from neutrality (Tajima's D test and Fu and Li's D test) were estimated in DNASP 4.10.8 (Tajima, 1989; Fu & Li, 1993; Rozas and Roses, 2006). A minimum spanning network of haplotypes was constructed in ARLEQUIN 3.0 (Excoffier, Laval & Schneider, 2005). Population differentiation of mtDNA variation was examined by estimating pair-wise F_{st} and testing for significance with 10 000 permutations in ARLEQUIN.

Results

Patterns of genetic variation at autosomal microsatellite loci

Five pairs of loci consisted of microsatellites located within 30 centimorgans of each other. From each of these pairs, we excluded the less informative locus (i.e. lower A and H_E), along with FCA262 because it was monomorphic. Autosomal genetic variation was examined among ocelots using the remaining 25 independent, variable loci (Cameron $n = 42$, Willacy $n = 28$, Mexico $n = 12$). Only one (FCA208) was out of HWE in all of the populations. FCA023 and FCA132 were out of HWE in Cameron, along with FCA035 in Mexico. Only 88% of the loci were polymorphic in Cameron, while all of the loci were variable in the other two populations (supporting information Table S3). The three populations had an excess of genetic diversity consistent with a recent bottleneck (Cameron $P = 0.01954$, Willacy $P = 0.00099$, Mexico $P = 0.02240$).

The highest levels of genetic diversity were observed in Mexico and intermediate levels in Willacy (Table 1 and supporting information Table S3). The A_R, H_O and H_S was

Table 1 Genetic diversity among 25 autosomal microsatellites and 395 bp portion of the mtDNA control region in three ocelot populations

	Autosomal microsatellites					MtDNA control region		
	n	*A*	A_E	A_P	H_E	*n*	π	D_{Hap}
Texas								
Cameron	42	2.88	2.00	6	0.399	39	0	0
1991–1998	29	2.84	2.00	6[a]	0.399	27	0	0
1999–2005	13	2.64	1.89	1[a]	0.379	12	0	0
Willacy	28	3.72	2.51	11	0.553	35	0.00064	0.252
1991–1998	18	3.66	2.52	22[b]	0.536	25	0.00082	0.324
2005	10	2.84	2.11	3[b]	0.480	10	0	0
Mexico								
Ebanos	12	4.64	3.30	42	0.637	15	0.00289	0.667

[a]With respect to the other Cameron temporal group

[b]With respect to the other Willacy temporal group

A, mean number of alleles; A_E, effective number of alleles; A_P, private alleles; H_E, expected heterozygosity; π, nucleotide diversity, D_{Hap}, haplotype diversity.

significantly lower in Cameron ($A_R = 1.841$, $H_O = 0.402$, $H_S = 0.407$) compared with Mexico ($A_R = 2.516$, $H_O = 0.605$, $H_S = 0.669$; $P = 0.0009$, 0.0018, 0.0009, respectively), while only H_O was significantly lower ($P = 0.0275$) compared with Willacy ($A_R = 2.131$, $H_O = 0.561$, $H_S = 0.528$). The differences between Willacy and Mexico were not significant. Mexico had roughly 5.5-fold greater number of private alleles than the two Texas populations despite a much smaller sample size.

In the AMOVA, the overall $F_{st} = 0.180$ was significant ($P = 0.001$), as were all pair-wise values. The highest was between Cameron and Mexico ($F_{st} = 0.272$, $P = 0.001$), and the lowest between Willacy and Mexico ($F_{st} = 0.113$, $P = 0.001$). The F_{st} between the two Texas populations was 0.163 ($P = 0.001$). All three populations also showed significant levels of differentiation in the genic and genotypic tests (X^2 = infinity, $P < 0.0001$ for all pair-wise comparisons in both tests). The N_em estimate was 0.444 per generation between all pairs of populations. There were no mis-assignments and no migrants detected among the three populations.

Model-based clustering without regard for geographic origin consistently found $K = 4$ clusters using Pritchard, Stephens & Donnely (2000) criteria and $K = 3$ clusters using the Evanno, Regnaut & Goudet (2005) method (Fig. 2a). In both cases, all cluster assignments were consistent with the population of origin. There were no clusters composed of individuals from more than one population. When $K = 4$, Willacy was split into two temporal groups, with one consisting of individuals captured in 1991–1998, and the other of those captured 2005. In analyses that tested $K = 2$, one cluster was comprised exclusively of ocelots from Cameron and the second cluster consisted of individuals from both Willacy and Mexico.

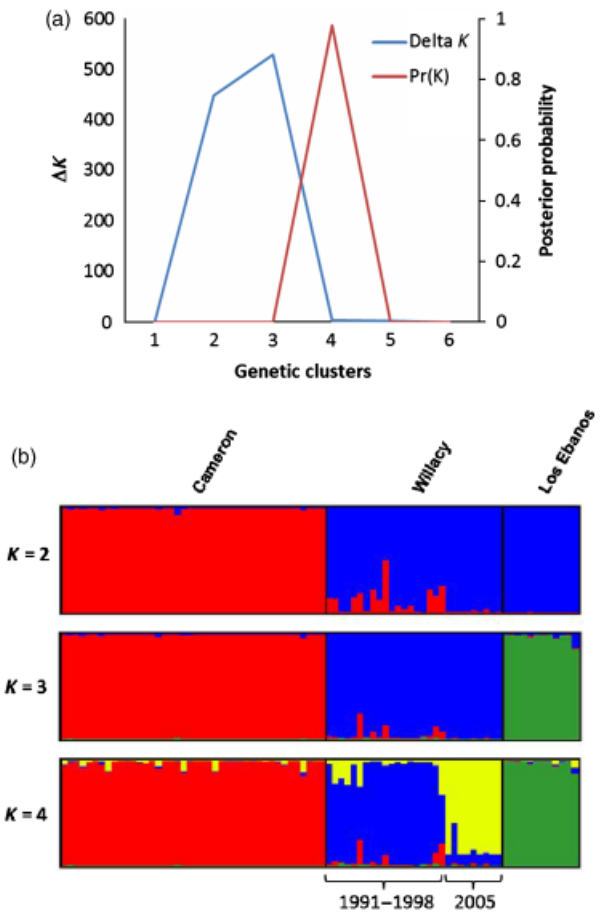

Figure 2 (a) Graph of the posterior probability of each cluster estimated in STRUCTURE and the change in likelihood derived using Evanno, Regnaut & Goudet (2005). The peaks represent the most likely number of clusters using the respective approach. (b) The proportion of individual variation for $K=2$, $K=3$ and $K=4$ assigned to a given genetic cluster in STRUCTURE. Each cluster is represented by a different color.

Assignment of individuals outside of main populations

Among the two individuals captured and radio-collared outside of the main populations, the one near Port of

Table 2 Population assignments using the Bayesian method in STRUCTURE and GeneClass for ocelots that did not originate in the three primary study areas

					P (STRUCTURE)			*P* (GeneClass)		
Date Collected	County	Location	mtDNA hap	Loci	Ca	Wi	Eb	Ca	Wi	Eb
Road-killed										
11/16/1989	Willacy	Port Mansfield	1	16	0.06	0.92	0.02	0.00	0.46	0.10
1989	Willacy	Port Mansfield	1	16	0.58	0.34	0.08	0.00	0.02	0.00
1989	Willacy	Port Mansfield	1	15	0.30	0.65	0.05	0.00	0.20	0.01
7/29/1991	Willacy	Port Mansfield	1	16	0.03	0.96	0.02	0.00	0.34	0.00
Oct 1993	Willacy	Port Mansfield	1	15	0.04	0.95	0.02	0.00	0.59	0.00
1/12/2004	Willacy	Port Mansfield	3	15	0.15	0.82	0.02	0.00	0.22	0.00
10/27/1997	Willacy	Lyford	1	25	0.02	0.97	0.01	0.00	0.27	0.00
6/17/1999	Willacy	Highway 186	1	15	0.25	0.74	0.01	0.00	0.03	0.00
4/7/1997	Cameron	Rio Hondo	1	15	0.67	0.20	0.13	0.00	0.00	0.00
10/15/1997	Kenedy	Sarita	1	16	0.33	0.63	0.03	0.00	0.09	0.00
8/31/1990	Kenedy	Sarita	1	15	0.28	0.70	0.03	0.00	0.16	0.00
Radio-collared										
5/8/1992	Hidalgo	SANWR	1	30	0.01	0.01	0.98	0.00	0.00	0.00
4/27/1998	Cameron	Port of Brownsville	1	15	0.97	0.02	0.01	0.99	0.00	0.00

Individuals from Texas were road-kills; except for ocelots from Santa Anna National Wildlife Refuge (SANWR) and Port of Brownsville which were live-trapped.
Ca, Cameron; Wi, Willacy; Mx, Mexico; *P*, probability.

Brownsville was assigned to Cameron, and the one in Santa Anna NWR most likely originated from Mexico. Eight of the 11 road-killed ocelots were assigned to the Willacy population (Table 2), including individuals found in Port Mansfield, Sarita and Lyford. Only one of the individuals from Port Mansfield could not be assigned, but had the highest probability of coming from Willacy. There was one road-kill found close to LANWR (i.e. Rio Hondo) that likely came from that population, although it could not be assigned with confidence.

Y-microsatellite diversity

Variation for Y-linked microsatellite loci was determined for 53 male ocelots (Cameron $n = 22$, Willacy $n = 18$, Mexico $n = 3$). Only two of the 12 loci were variable among the individuals examined and both were located in the SMCY gene, although in different introns (SMCY2 in intron 2 and SMCY7 in intron 7). Four alleles were present SMCY2 (168, 169, 172 and 180) and six in SMCY7 (167, 169, 171, 173, 175 and 177). However both loci exhibited a large number of what appeared to be heterozygous genotypes with the shorter allele having a higher peak than the longer allele (nine heterozygotes in SMCY2 and 12 in SMCY7). As the Y-chromosome is haploid, this suggests that the locus may have been duplicated in ocelots. Because of this inconsistency we did not use the Y microsatellites in our analysis of genetic diversity.

Mitochondrial diversity and differentiation

A 395-bp fragment of the control region was sequenced for 77 ocelots (Cameron, $n = 39$; Willacy, $n = 35$; Mexico, $n = 15$). There were four haplotypes (Hap) with three variable sites (Fig. 3), all of which were present in Mexico [frequencies = 0.533 (Hap 1), 0.067 (Hap 2), 0.133 (Hap 3) and 0.267 (Hap 4); GenBank accessions JF930139–JF930142]. Hap 1 also occurred in Cameron (1.00) and Willacy (0.857), however, in Texas Hap 2 was only found in Willacy (0.143). Haps 3 and 4 were not detected in any individuals from Texas. Overall, Mexico had the highest levels of haplotype and nucleotide diversity, followed by Willacy, with no diversity detected in Cameron (Table 1, Fig. 1).

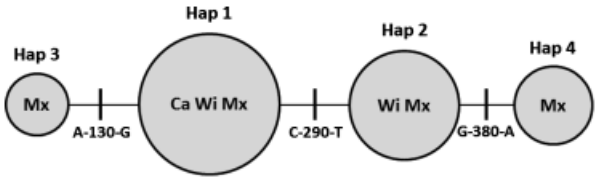

Figure 3 Haplotype (Hap) map of 395-bp portion of the MtDNA control region. Each hatch mark represents a single nucleotide substitution with the specific nucleotide states given. The size of each haplotype circle is based on its frequency among all ocelots, although sizes are not directly proportional [frequencies = 0.877 (Hap 1), 0.066 (Hap 2), 0.019 (Hap 3) and 0.038 (Hap 4)]. Ca, Cameron; Wi, Willacy; Mx, Mexico.

The greatest level of mtDNA differentiation was between the Cameron and Mexico populations with an F_{st} value of 0.451 ($P < 0.001$), and significantly different haplotype frequencies. The lowest level of differentiation was between Willacy and Cameron ($F_{st} = 0.126$, $P = 0.018$). The F_{st} between Willacy and Mexico was 0.180 ($P = 0.005$). A rapid

loss of diversity was observed in Willacy over a span of only 7 years. In this population, D_H was 0.324 among samples collected 1991–1998, yet in 2005 there was no diversity detected in the mitochondrial control region.

Discussion

Genetic variation within and between ocelot populations

Lowest diversity was observed in ocelots from Cameron, which is the only breeding population occurring on a US federal or state refuge. The level of microsatellite diversity at LANWR was comparable to variation in felid populations that have recently undergone severe bottlenecks, including the critically endangered Amur leopards *Panthera pardus oreintalis* in the Russian Far East and North Korea (Uphyrkina *et al.*, 2002), and those that have been recently isolated by anthropogenic factors, such as mountain lions in the central coast and southern regions of California (Ernest *et al.*, 2003). In addition, no variation was observed for the control region segment in the Cameron population. This is consistent with the small N_e estimates for the time period spanning 1989–1996, ranging from 8.0 to 13.9 (Janečka *et al.*, 2008). The small effective population size coupled with its isolation has increased the effects of drift and inbreeding leading to low genetic diversity compared with Willacy and Tamaulipas.

The Willacy population retained more ancestral variation and was less divergent from Mexico based on estimates of genetic differentiation and the grouping of Willacy and Mexico into one cluster for $K = 2$ (Fig. 2b). Yet, the N_e of Willacy between 1998 and 2005 was estimated to be smaller than in Cameron (maximum estimate of 3.1) despite the intermediate levels of genetic diversity (Janečka *et al.*, 2008). In the cluster analysis of autosomal variation for $K = 4$ there was a division of Willacy into two distinct genetic groups corresponding to ocelots captured in the 1990s and those in 2005. Over that period, there was also a loss of 22 autosomal microsatellite alleles and the mtDNA data shows a reduction from moderate haplotype diversity during mid-1990s, to no diversity in 2005. This suggests that until the 1990s Willacy was likely larger and more widely distributed, but over the last decade has been subject to extreme genetic drift as a result of reductions that led to a very small population size and demographic instability. This scenario is also consistent with the observed patterns in heterozygosity that were indicative of a recent bottleneck. Although diversity was highest in northeastern Mexico, a recent reduction in effective size was also detected in that area, suggesting the Tamaulipas region may likewise be currently undergoing population reductions.

Genetic divergence

Both microsatellite and mtDNA analyses revealed significant genetic subdivision among the three populations. The F_{st} between Cameron and Willacy, which are separated by *c*. 30 km, was higher than between Willacy and Tamaulipas, which are more than 300 km apart. Historical gene flow can influence estimates of F_{st}; therefore, the lower value between the two geographically distant populations is likely the result of greater levels of ancestral variation retained by Willacy compared with the Cameron population, not contemporary gene flow. Estimates of N_em are below one individual per generation between all pairs of ocelot populations, thus indicating an overall lack of connectivity. A value of $N_em = 1$ is roughly considered the minimum for maintaining population connectivity sufficient to prevent genetic divergence (Mills & Allendorf, 1996).

Assignment tests reflect contemporaneous dispersal more precisely than F_{st} estimates because they allow for the identification of recent migrants (Rannala & Mountain, 1997; Paetkau *et al.*, 2004; Manel, Gaggiotti & Waples, 2005). There were no mis-assignments or migrants observed among the three ocelot populations. Furthermore, in the Bayesian analysis using STRUCTURE, all clusters were composed of individuals from only one population. The absence of mis-assignments and migrants, and complete resolution of populations into distinct clusters, illustrates the high levels of isolation and subsequent differentiation.

The extreme genetic divergence observed between proximate areas in Texas <30 km apart is unusual for a medium-sized carnivore. Felids are physically capable of long-distance dispersal (>100 km), and exhibit reasonable amounts of connectivity over large geographic areas (Hellborg *et al.*, 2002; Sunquist & Sunquist, 2002; Ernest *et al.*, 2003). The high amount of differentiation between the two ocelot populations is likely a result of habitat modifications in the Lower Rio Grande Valley, combined with small population sizes, and is analogous to the effects of anthropogenic changes on Iberian lynx *Lynx pardinus* in Spain and black bears *Ursus americanus* in Florida, where significant genetic structure in highly vagile species was linked to anthropogenic dispersal barriers (Johnson *et al.*, 2004; Dixon *et al.*, 2007).

Human activities in southern Texas and northeastern Mexico have eliminated large tracts of dense thornshrub communities preferred by ocelots (Tewes & Everett, 1986; Jahrsdoerfer & Leslie, 1988). The area between the Cameron and Willacy population is characterized by agricultural regions with greatly reduced native plant communities and extensive sorghum and cotton fields many kilometers wide (Fig. 4). The strong habitat selection of ocelots for dense thornshrub, and avoidance of areas with $<75\%$ canopy cover, likely reduces movement between relatively close habitat patches (Horne *et al.*, 2009). Our study suggests that this has resulted in complete isolation of the two remaining Texas ocelot populations. Absence of dispersal between the Cameron and Willacy populations strongly suggested from the genetic data is also supported by ecological research; no dispersal events have been observed between these two areas in nearly 30 years of radio-telemetry work (Tewes, 1986; Laack, 1991; Caso, 1994; Beltran & Tewes, 1995; Shindle & Tewes, 2000; Haines, Tewes & Laack, 2005; Laack *et al.*, 2005; Haines *et al.*, 2006*a*,*c*; Horne *et al.*, 2009).

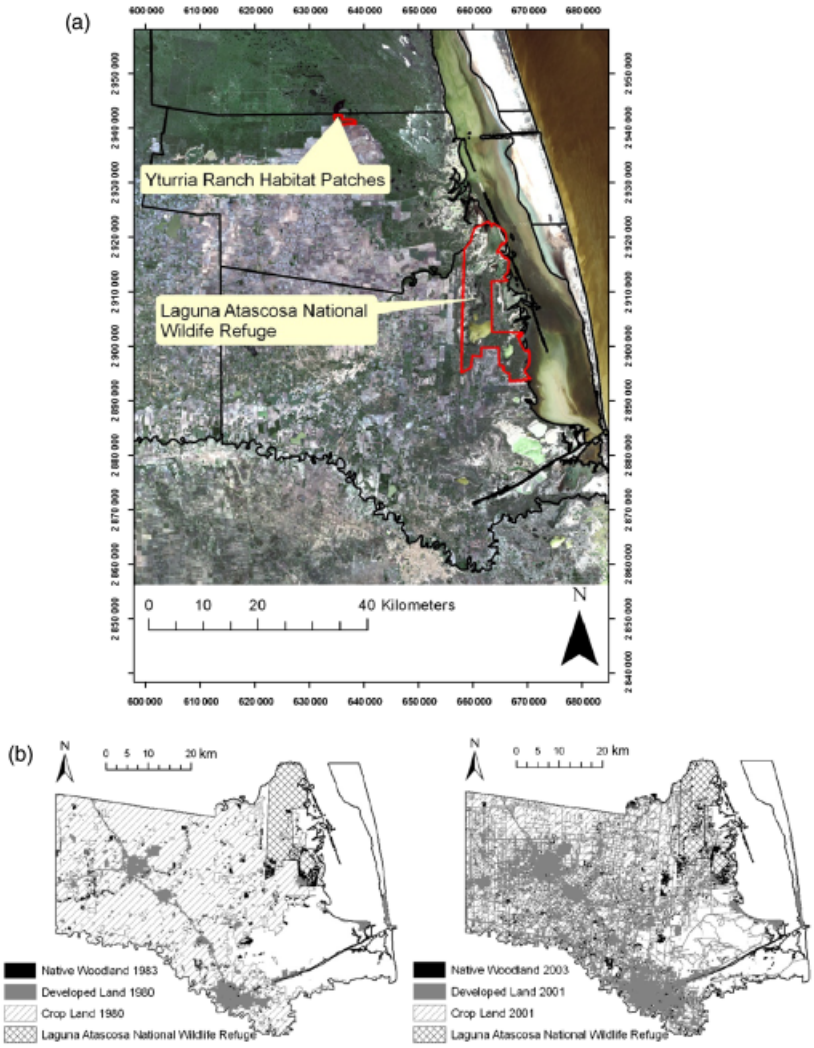

Figure 4 (a) Satellite image showing the distribution of agriculture in the Lower Rio Grande Valley. (b) Map illustrating the amount of development from 1980 to 2001 in Cameron County, Texas.

Road mortality a hindrance to ocelot dispersal

Vehicle collisions are the highest source of anthropogenic mortality for ocelots (Haines, Tewes & Laack, 2005), and further limit dispersal through the agricultural lands surrounding the remaining habitat patches. For instance, two road-killed ocelots found near LANWR were assigned to Cameron and seven road-kills to Willacy. Presumably these cats were moving out of their natal area, yet died in a vehicular accident before reaching the other US breeding population.

Ocelots from Port Mansfield, Sarita and Raymondville belonged to the Willacy population, suggesting that it may extend outside of its known distribution. Interestingly, one of the Port Mansfield road-kills collected in 2004 had mtDNA Hap 2, which was not detected in the 2005 Willacy sample. Native rangeland is present to the north, west and east of Willacy, which is likely more porous to ocelot movement compared with the agricultural landscape that borders the Cameron population (Fig. 4), thereby facilitating dispersal to the north and east. Willacy therefore is a critical source for natural recolonization of areas close to the Yturria habitat patches.

The only evidence for movement between Texas and Tamaulipas was the individual captured in SANWR in 1992, which was assigned to Mexico and also possessed a high number of private alleles from that population. Therefore, this ocelot likely came from habitat south of the Rio Grande River, suggesting the possibility that there is an unknown ocelot population closer to the US border than the one sampled in southern Tamaulipas. This individual is one of the few ocelots documented in SANWR since 1990, and potentially represents rare dispersal events that thus

far failed in reaching either of the US breeding populations. The ocelots sampled in Miradores and Los Zoyates were assigned to the Mexico population.

Implications for conservation

Ocelot reached the northern extent of their historical range in parts of Texas, Louisiana and Arkansas (Lowery, 1974). This region, along with Tamaulipas, together constitute a closely related phylogenetic clade suggesting former connectivity likely maintained via thornshrub habitat that was previously more widely distributed (Janečka *et al*., 2007; Jahrsdoerfer & Leslie, 1988). In the past, Mexico may have served as a source for populations in the US. Any potential metapopulation dynamics have now been disrupted by brush removal, row crop agriculture, road development and urbanization that have greatly altered the landscape (Fig. 4). These barriers impede ocelot movement between the few remnant habitat patches. Our genetic data strongly indicate that habitat fragmentation has resulted in virtually complete isolation of the two extant populations in Texas, in addition to an overall loss of genetic variation.

Therefore, the extinction rate for both Texas populations likely exceeds the rate of colonization, and the fates of each population in Texas are independent from each other as well as from existing populations in Mexico. Both populations are well below the minimum population viability size recommended for long-term survival (Franklin, 1980; Shaffer, 1981; Reed *et al*., 2003). As shown across diverse taxa, including several species of felids, a continued loss of genetic diversity through increased drift and inbreeding as the number of potential breeders declines contributes to reductions in fitness and greater extinction risks (Roelke, Martenson & O'Brien, 1993; Frankham & Ralls, 1998; Hedrick & Kalinowski, 2000; Keller & Waller, 2002; Reed & Frankham, 2003; Reed, Nicholas, & Stratton, 2007, Johnson et al., 2010). Research is needed to determine if inbreeding depression is affecting ocelots in Texas.

Conservation actions can be implemented to offset the continued decline of ocelot populations in the US. Development of habitat corridors in conjunction with safe passages across highways associated with mortality of dispersing ocelots would facilitate exchange between populations, improving the overall stability of the Texas populations (Haines *et al*., 2006*c*). However, the Lower Rio Grande Valley is a rapidly growing area (Fig. 4b), and the creation of such corridors is not logistically feasible. Translocations represent the only practical method for reconnecting these populations, although the potential effects must be first carefully evaluated (Hedrick, 2010).

In addition to the demographic benefits of translocations, genetic variation in Texas could be partially restored and inbreeding reduced, minimizing the potential for inbreeding depression (Johnson et al., 2010; Hedrick, 2010). Populations of *L. p. albescens* from Tamaulipas are a genetically appropriate source based on their phylogenetic relationship, and would yield the highest increase in variation (Janečka *et al*., 2007). However, it also would be beneficial to move individuals between Cameron and Willacy. There is significant divergence and high number of private alleles when these populations are compared with each other, suggesting each has preserved a different portion of ancestral variation. A partial reconstruction of historical levels of diversity could be achieved by mixing the Texas populations. Finally, expansion of ocelots into isolated vacant habitat patches will likely also require translocations. These management strategies should enhance the long-term viability of the remaining ocelot populations in Texas.

Acknowledgments

We thank F. Yturria, M. Corbett and C. Corbett for access to their ranches. Logistical support was provided by F. Yturria in Texas and Los Ebanos Ranch in Mexico. This study was funded by the Rob and Bessie Welder Wildlife Foundation, Tim and Karen Hixon Foundation and F. Yturria. This article represents publication number 700 of the Rob and Bessie Welder Wildlife Foundation and article number 11–122 of the Caesar Kleberg Wildlife Research Institute at Texas A&M University-Kingsville.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Ocelots sampled in the US and Mexico that were used for population structure analysis in this study. Ten additional ocelots were genotyped for Y microsatellites but were excluded from this table because they were not used to asses autosomal and mtDNA diversity.

Table S2. The chromosomal position of 41 autosomal microsatellites in the domestic cat genetic linkage and radiation hybrid maps that were screened in ocelots (NCBI Map Viewer Build 0.1, Menotti-Raymond *et al.*, 2003*a*,*b*; 2009; Davis *et al.*, 2009). Microsatellites in bold were selected for population structure analysis. PCR results (res.) are coded as; + = robust amplification, − = no amplification, and M.A. = multiple amplicons of different size. PCR conditions (cond.) refer to whether the primers were fluorescently labeled directly on the 5′ end (1) or using a dye-labeled m13 tag (2).

Table S3. Genetic diversity among 25 autosomal microsatellites in three ocelot populations sampled from 1991–2005. Abbreviations: Ca = Cameron County, Texas; Wi = Willacy County, Texas; Mx = Mexico; H_O = observed heterozygosity, H_E = expected heterozygosity; A_N = effective alleles.

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